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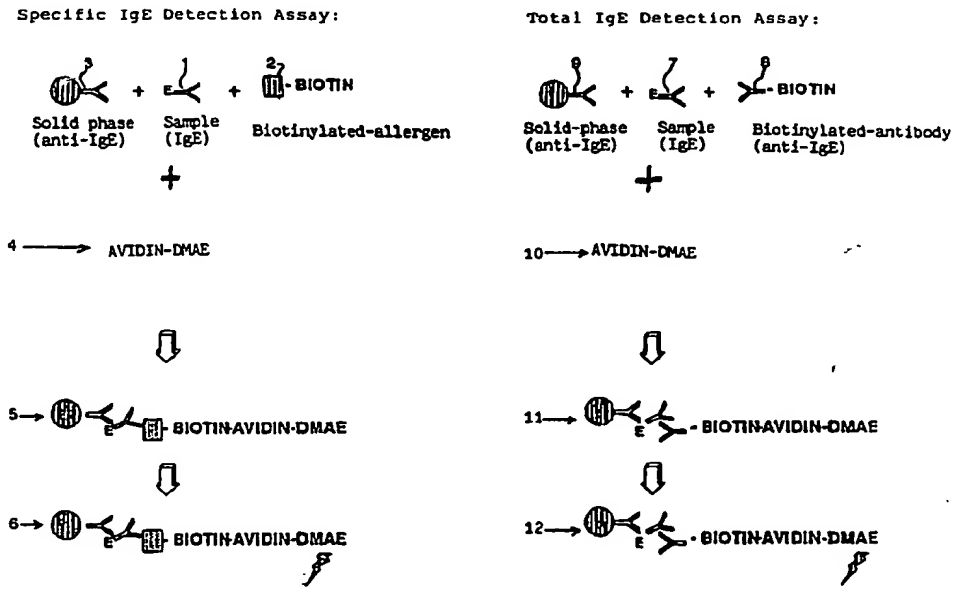

(57) Abstract

A method of detecting an antibody in a sample using a labelling compound and comprising the steps of mixing a ligand antigen, antibody or hapten bound to biotin with the sample; an antibody directed against the antibody to be detected bound to paramagnetic particles; and a chemiluminescent acridinium compound bound to avidin or streptavidin to form a solid phase complex; separating the solid phase from the liquid phase; and analysing the separated solid phase for the presence of chemiluminescent complex.

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TWO-SITE IMMUNOASSAY FOR AN ANTIBODY WITH CHEMILUMINESCENT LABEL AND BIOTIN BOUND LIGAND

The present invention relates to a method of detecting an antibody in a sample using a chemiluminescent labelling compound.

More specifically, the invention relates to the use of a chemiluminescent acridinium ester compound coupled to avidin or streptavidin, and a ligand coupled to biotin in a two-site immunoassay wherein the affinity complex is captured on paramagnetic particles, which makes possible a rapid detection and/or quantification of immunologically active substances, such as antibodies in samples such as biological fluids and tissue samples, milk, food samples, beverages, water or industrial effluents.

A method of detecting and quantifying immunoglobulin E-antibodies in serum is disclosed in the brochure "Specific IgE, Magic® Lite SQ™, published by Ciba Corning Diagnostics Corp. and ALK Laboratories in September 1990. In said method a specific allergen covalently bound to paramagnetic particles reacts with the allergen-specific IgE-antibody in a serum or plasma sample. After a first incubation period and washing away unbound non-specific IgE, a chemiluminescent acridinium ester-labelled monoclonal antibody against IgE is added. Following a second incubation period the solid phase bound and labelled antibody is measured in a Magic Lite® Analyzer (Cat. No. 472733 or Cat. No. 472270) which automatically injects reagents, which initiate the chemiluminescent reaction. When using said method only the final step of initiating and measuring the chemiluminescence can be automated. The chemiluminescent acridinium ester labelling compound is described in US patent No. 4,745,181.

US patent No. 4,946,958 discloses a chemiluminescent acridinium ester linked to an N-succinimidyl moiety which can be further linked to a protein or polypeptide to provide an immunologically reactive luminescent reagent. Said reagent can be used in an immuno-assay, which may involve the simultaneous binding of a solid phase antibody, an antigen molecule and a labelled anti-antibody, separation and washing of the solid phase and quantifying the luminescence of the solid phase.

Strasburger et al. discloses in Methods in Enzymology, 184(1990), pp 481-496 the use of a two-site chemiluminescent immuno-assay, wherein hGH and hCG hormones are captured by antibodies immobilized on microtiter plates and labelled with a chemiluminescent agent coupled to avidin through a biotin labelled second antibody. Antigenic analytes, such as protein hormones, may be assayed directly from serum samples and compared to standard curves. The concentration of immunoglobulins, such as IgE, is, however, extremely patient dependent and an assay of a specific IgE must be compared with the individual patient level of total IgE and therefore reuqires an assay having a greater dynamic range than is obtainable in the assay disclosed by Strasburger et al.

EP-A-0 425 217 discloses a hybridization assay wherein a chemiluminescent complex is formed comprising a nucleic acid hybridised with a first labelled nucleotide probe coupled to paramagnetic particles and a second nucleotide probe labelled with biotin and coupled to an avidin-acridinium ester. However, the person skilled in the art confronted with the problem of providing a fully automated method of detecting antibodies will search for a method which can be carried out in one reaction container and preferably under ambient reaction conditions. The assay represented here is fundamentally different as it employs detection of specific nucleotide sequences, which are not antigens towards which specific antibodies can be raised. More detailed it is necessary in said assay to use elevated temperatures for the hybridization and a hapten-oligonucleotide probe which is not necessary nor desirable in the immuno-assays.

Until now immuno-assays for the quantification of immunologically active molecules, such as immunoglobulins (e.g. specific immunoglobulin-E), in biological fluids, such as serum, have been manual, e.g. the Enzyme Linked Immuno Sorbent Assay (ELISA), or semi-automatical. And the typical duration of a semi-automatical immunoassay, such as the Magic® Lite SQ™ specific IgE assay referred to above, is approximately two hours.

Moreover, commercial specific IgE assays (CAP,RAST, supplied by Pharmacia, Uppsala) and the Magic® Lite SQ™ specific IgE assay use

a total-IgE reference assay having a non-identical protocol resulting in unprecise data. Only assays using the same catching and detection procedures are directly comparable. For example all specific IgE close response curves must be parallel with total IgE response curves. The reason is that the required dynamic range for a specific (or total?) IgE assay is 2 decades and the required dynamic range for a total IgE assay is from 2 to 7 decades, and the existing immuno-assays do not allow concentration measurements over the entire range. Thus, until now it has been necessary to use different protocols for the specific and total immunoglobulin assays having different reagents.

Because of the increasing interest in safe laboratory procedures there is a need for a fully automated method of detecting substances, such as antibodies, in biological fluids, such as human serum, plasma, blood, milk, urine or saliva, which method should provide a minimised risk of contact with hazardous fluids. Also, the increasing use of laboratory tests in diagnostics calls for methods of short duration, preferably only a few minutes.

It is therefore an object of this invention to provide a method of detecting an antibody in a sample, which method is safe, rapid, and fully automatable.

This object is achieved by one method according to the invention which method is characterised in:

a) mixing a ligand antigen, antibody, or hapten bound to biotin or a functional derivative thereof; an antibody directed against the antibody to be detected bound to paramagnetic particles; and a chemiluminescent acridinium compound bound to avidin, streptavidin or a functional derivative thereof with the sample to form a solid phase bound complex,

b) magnetically separating the solid phase from the liquid phase,

c) initiating the chemiluminescent reaction, and analysing the separated solid phase for the presence of chemiluminescent complex, wherein the presence of chemiluminescence is an indication of the

presence of said antibody in said sample.

Although the method according to the invention can be carried out by the above defined steps (a), (b) and (c) it is preferred to add the labelling compound in a separate step, and the method is preferably carried out according to the following steps:

i) mixing the ligand antigen, antibody, or hapten bound to biotin or a functional derivative thereof with the sample and the antibody directed against the antibody to be detected bound to paramagnetic particles to form a first solid phase complex,

ii) adding a chemiluminescent acridinium compound covalently bound to avidin, streptavidin or a functional derivative thereof to form a second solid phase complex,

iii) magnetically separating the solid phase from the liquid phase;

iv) initiating the chemiluminescent reaction, and analysing the separated solid phase for the presence of chemiluminescent complex.

A particular object of the invention is to provide a fully automatable immuno-assay for the quantification of specific antibodies, such as immunoglobulins, wherein a truly parallel reference immuno-assay using an identical protocol is used as the reference.

The object of quantifying specific antibodies using a truly parallel reference immuno-assay is achieved by a method of measuring the concentration and/or the relative contents of a specific antibody in a liquid sample, wherein the measured light emission of a separated solid phase comprising a captured specific antibody coupled to a chemiluminescent label is compared with the measured light emission obtained in a truly parallel reference immuno-assay wherein the total contents of the class of antibodies in the sample to which said specific antibody belongs is measured. The quantification of the specific antibody is preferably achieved by a method wherein the light emission of the separated solid phase comprising the captured specific antibody coupled to a chemiluminescent label is obtained by

a) mixing a ligand antigen or hapten towards which the specific antibody to be measured is directed bound to biotin or a functional derivative thereof; an antibody directed against the constant portion of the antibody to be measured bound to paramagnetic particles; and a chemiluminescent acridinium compound bound to avidin, streptavidin or a functional derivative thereof with the sample to form a solid phase complex,

b) magnetically separating the solid phase from the liquid phase,

c) initiating the chemiluminescent reaction and measuring the light emission,

and the reference immuno-assay for the class of antibodies to which said specific antibody belongs comprises

a) mixing a ligand antibody directed against said class of antibodies to be measured bound to biotin or a functional derivative thereof; an antibody directed against the constant portion of the class of antibodies to be measured bound to paramagnetic particles; and a chemiluminescent acridinium compound bound to avidin, streptavidin or a functional derivative thereof with the sample to form a solid phase complex,

b) magnetically separating the solid phase from the liquid phase,

c) initiating the chemiluminescence reaction and measuring the light emission.

Another preferred method is a method wherein the light emission of the separated solid phase comprising the captured specific antibody coupled to a chemiluminescent label is obtained by

i) mixing the ligand antigen or hapten towards which the specific antibody to be measured is directed bound to biotin or a functional derivative thereof with the sample and the antibody directed against the constant portion of the antibody to be measured bound to paramagnetic particles to form a first solid phase complex,

ii) adding a chemiluminescent acridinium compound covalently bound to avidin, streptavidin or a functional derivative thereof to form a second solid phase complex,

iii) magnetically separating the solid phase from the liquid phase,

iv) initiating the chemiluminescent reaction, and measuring the light emission,

and the reference immuno-assay for the class of antibodies to which said specific antibody belongs comprises

i) mixing a ligand antibody directed against said class of antibodies to be measured bound to biotin or a functional derivative thereof with the sample and the antibody directed against the constant portion of the class of antibodies to be measured bound to paramagnetic particles to form a first solid phase complex,

ii) adding a chemiluminescent acridinium compound covalently bound to avidin, streptavidin or a functional derivative thereof to form a second solid phase complex,

iii) magnetically separating the solid phase from the liquid phase,

iv) initiating the chemiluminescent reaction and measuring the light emission.

The specific antibody to be measured in the sample is preferably a specific immunoglobulin selected from the group consisting of IgA, IgD, IgE, IgG, IgM, and isotypes thereof, and the ligand antigen, antibody, or hapten directed against the variable portion of said antibody is an allergen, and the class of antibodies is preferably a class of immunoglobulins selected from the group consisting of total IgA, total IgD, total IgE, total IgG, total IgM, and isotypes thereof, and the ligand antigen, antibody or hapten is an antibody directed against said class of immunoglobulins.

More preferably the specific immunoglobulin is a specific IgE, and the class of antibodies is total IgE.

The antibody directed against the antibody to be measured bound to paramagnetic particles is preferably selected from the group consisting of polyclonal antibodies, monoclonal antibodies including recombinant antibodies, fragmented antibodies, preferably a monoclonal mouse anti-immunoglobulin.

The advantages of the invention are:

- All reagents can be mixed simultaneously in one reaction container, which minimises the risk of contamination, errors and operation steps, reduces significantly the duration of the immuno-assay, and greatly facilitates automation of the process and precision is improved, cf. example 6;

- Quantification of specific antibodies in, e.g. a serum sample, can be performed with reference to a truly parallel total antibody assay using an identical protocol, cf. example 3;

- The obtained greater capacity and sensitivity facilitates that even very low concentrations of immunoglobulins and low concentrations of specific immunoglobulins can be detected, cf. examples 1, 2 and 7.

A preferred embodiment of the invention is an immuno-assay for the detection of antibodies, such as specific immunoglobulins (IgA, IgE, IgG, IgM and isotypes thereof) in a sample, such as serum or saliva.

Particularly, the invention can be used in an assay for the detection and quantification of specific IgE in a sample. When the sample is liquid, e.g. serum or plasma, it can be added directly to a reaction container comprising preferably a monoclonal mouse anti-IgE antibody bound to suspended paramagnetic particles and a specific allergen (ligand) bound to biotin in an aqueous medium. The biotin is preferably biotin amidocaproate N-hydroxysuccinimide ester (Sigma Catalog No. B2643) When the sample is non-liquid, e.g. a tissue sample, it is preferably homogenised and suspended in an aqueous liquid. A simultaneous reaction between specific IgE in the sample, allergen and monoclonal anti-IgE antibody in the aqueous medium results in the formation of a conjugate. A chemiluminescent la-

belling compound, preferably an acridinium ester coupled to streptavidin (Sigma Catalog No. S4762) (avidin-DMAE) is added to the reaction container and a binding reaction between avidin-DMAE and biotin bound to the conjugate and unconjugated allergen-bound biotin takes place. The conjugate bound label is separated from the unbound DMAE-labelled antibody by magnetically separating the reaction mixture and decanting the supernatant. The chemiluminescence of the separated conjugate is measured as described in Pazzagli, M. et al. (eds.). Studies and applications in "Biology & Medicine", Journal of Bioluminescence and Chemiluminescence 4(1), 1-646, 1989.

As a reference, a truly parallel total-IgE immuno-assay differing only in that a preferably polyclonal anti-IgE antibody bound to biotin is used as the ligand is performed simultaneously.

Figure 1 is a diagrammatic representation of a specific IgE assay according to the invention and comprising a parallel total IgE reference assay. In the figure (1) represents the specific IgE-antibody to be detected, (2) is a specific allergen bound to biotin, (3) is a monoclonal mouse anti-IgE bound to paramagnetic particles, (4) is an avidin-acridinium ester and (5) represents the solid phase labelled complex formed between (1), (2), (3) and (4) and includes optional incubation, separation and optional washing steps, and (6) represents a final step of initiating the chemiluminescent reaction and measuring the light emission.

In the total IgE reference assay in Figure 1 (7) represents IgE (WHO 75/502 IU/ml), (8) is polyclonal anti-IgE bound to biotin, (9) is monoclonal mouse anti-IgE bound to suspended paramagnetic particles, (10) is an avidin-acridinium ester and (11) represents the solid phase labelled complex formed between (7), (8), (9) and (10) and includes optional incubation, separation and optional washing steps, and (12) is the final step of initiating the chemiluminescent reaction and measuring the light emission.

More particularly, the immuno-assay using the method of the invention can be performed in ACS:180 fully automatic analyzer produced by Ciba Corning Diagnostics Corp., Medfield, Mass., U.S.A.

In one preferred embodiment of the invention the immunologically active substance to be detected is an antibody, e.g. against penicillin or derivatives thereof, such as benzylpenicillin, penicilloyl, etc., and the ligand bound to biotin is a hapten, such as penicillin or derivatives thereof.

Definitions

In the methods of the invention the antibody to be detected is a specific immunoglobulin, preferably a specific IgA, IgD, IgE, IgG, IgM, and isotypes thereof, and more preferably a specific IgE, or a class of antibodies, such as immunoglobulins, preferably selected from the group consisting of total IgA, total IgD, total IgE, total IgG, total IgM and isotypes thereof, most preferably total IgE.

By sample is meant any liquid or liquefied sample, including solutions, emulsions, dispersions and suspensions.

The ligand antigen, antibody or hapten bound to biotin can be any immunologically active substance, such as an allergen, antibodies, such as polyclonal antibodies, monoclonal antibodies including recombinant antibodies or fragmented antibodies, preferably an allergen and/or a polyclonal anti-immunoglobulin, such as goat anti-human polyclonal serum spplied by Ventrex Laboratories, Inc., Portland, Maine, Catalog No. 77660. In the reference immuno-assay said antibody is preferably directed against the constant portion of the class of antibodies to be measured, i.e. an antibody directed against the IgE-antibodies.

By biological fluid is meant any clinical sample, such as blood, plasma, serum, urine or saliva, which also includes any biological fluid which is excreted, secreted or transported internally in an organism.

By paramagnetic particles (PMP) is meant particles which can be dispersed or suspended in a liquid medium. Throughout the examples are used BioMag particles (iron oxide particles coated with amine terminated groups) sold by advanced Magnetics Inc., Cambridge, Massachusetts. The antibodies coupled to PMP are preferably directed

against the constant portion of the antibodies to be detected or measured and may be polyclonal or monoclonal antibodies including recombinant or fragmented antibodies, preferably a monoclonal antibody, MAb A 5697-1A3(920325) supplied by BioInvent International AB, Lund, Sweden.

The chemiluminescent acridinium compound is preferably N-hydroxy-succinimide dimethylacridinium ester covalently bound to avidin or streptavidin (avidin-DMAE). Avidin and DMAE are coupled according to the methods of Weeks et al., Clin. Chem. 29/8, 1474-1479 (1983). Other luminescent labelling compounds that can be bound to avidin or streptavidin may be used in the method of the invention. E.g. luminol, lucigenin or lophine.

Preparation of biotinylated antibodies

Biotinylated anti-IgE and Phleum pratense:

Goat anti-human polyclonal serum (Ventrex Laboratories, Inc. MA, USA) is purified by affinity chromatography on a CNBr-activated sepharose 4B (Pharmacia, Uppsala, Sweden) with myeloma IgE (OEM concepts, USA) as a ligand. The anti-IgE is biotinylated with the ratio mol biotin: mol anti-IgE = 41:1.

9 µl of Biotin (Biotin amidocaproate N-hydroxysuccinimide ester (Sigma) 25 mg/ml in Dimethylformamide (Merck) is added to 0.4 ml of anti-IgE 4.5 mg/ml in 0.1 M $NaHCO_3$ (Merck). The reagents are incubated in an "end over end" mixer for 2 hours at 25°C. 0.9 ml lysin (Sigma) solution 20 mg/ml $NaHCO_3$ is added. The solution is filtered and the biotinylated antibody is purified by size chromatography on superdex 75 Hiload 16/60 (Pharmacia, Uppsala, Sweden). The pooled fractions are diluted in phosphate buffered saline PBS, pH 7.2, containing 0.1 % human serum albumin (Sigma) 0.1 % NaN_3 (Sigma).

The Phleum pratense extract, (ALK Laboratories A/S, Hørsholm, Denmark) is biotinylated in the molar ratio of 10:1 0.65 ml of biotin 10 mg/ml is added to 0.43 ml of 10 mg/ml Phleum pratense in 0.1 M $NaHCO_3$. The reagents are incubated for 2 hours at 25°C in an

"end over end" mixer, after the incubation 40 µl lysin (Sigma) solution 50 mg/ml is added. The solution is filtered and the biotinylated antibody is purified from excess of biotin by size exclusion chromatography on superdex 75 Hiload 16/60 (Pharmacia). The fractions containing the allergens are pooled. The biotinylated Phleum pratense is diluted with PBS pH 7.2, containing 0.1 % human serum albumin (Sigma) and 0.1 % NaN_3 (Sigma).

Preparation of streptavidin-acridinium ester label

Streptavidin was conjugated with DMAE-NHS, [2',6'-dimethyl-4'-(N-succinimidyloxycarbonyl)phenyl-10-methylacridinium-9-carboxylate methosulphate] using the methods of Weeks et al., Clin.Chem. 29/8, 1474-1479 (1983).

Preparation of Streptavidin-acridinium ester label:

0.96 mg N-hydroxysuccinimide dimethylacridinium ester DMAE (Ciba Corning Diagnostics Corp., Medfield, MA, USA) is diluted in 1.92 ml Dimethylformamide. 250 µl of this solution is pipetted to 2.5 ml 1 mg/ml streptavidin (Sigma) in 0.1 M sodium dihydrogenphosphate, 0.15 M NaCl pH 8.15.

The air above the solution in the vial is exchanged with nitrogen, (AGA). The reagents are incubated for 30 min at 25°C under stirring, after incubation 2250 µl 10 mg/ml lysin 0.1 M sodium dihydrogen-phosphate (Merck), 0.15 M NaCl is (Merck) pH 8.15 is added. To remove unbound DMAE the solution is loaded on a PD-10 column (Pharmacia, Uppsala, Sweden). The eluate is collected and purified by ultrafiltration using a cellulose Minitan-S filter sheet 10.000 NMWL (Millipore). The filtration is performed with 1.5 l phosphate buffered saline, PBS pH 7.2. The retentate (retanate) is concentrated to 25 ml and 25 ml PBS pH 7.2 containing 0.5 % HSA (Sigma) and 0.1 % NaN_3 (Sigma) is added.

The invention will be described in detail in the following examples.

Immobilisation of antibody to paramagnetic particles

6.5 g paramagnetics particles (Ciba Corning Diagnostics Corp., MA, USA) are washed in 650 ml methanol (Merck) 3 times using magnetic separation. A wash with 650 ml 0.01 M acetate buffer pH 5.5 is performed twice. The particles are activated in 6.25 % glutaraldehyde (Merck), 0.01 M acetate buffer pH 5.5 for 3 hours at 25°C. The particles are washed 3 times in 650 ml 0.01 M acetate buffer pH 5.5. The particles are coupled with 1083 mg monoclonal anti-IgE antibody (ALK Laboratories, Hørsholm, Denmark) specific against the IgE Fc domain, for 24 hours at 25°C. The particles are washed twice in 0.01 M acetate buffer pH 5.5. Blocking of excess of active groups is performed with 200 ml 10 % IgE stripped serum (ALK Laboratories, Hørsholm, Denmark) for 24 hours at 25°C. The particles are washed in 650 ml 0.01 M phosphate buffer (Merck) followed by 3 washes in 650 ml 1 M NaCl (Merck). The particles are washed 3 times in 0.01 M phosphate buffer. The particles are resuspended in 650 ml PBS pH 7.2, 0.1 % w/v bovine serum albumine (Sigma), 0.001 % bovine gamma globulin (Sigma) and heat treated for 18 hours at 50°C. The particles are washed 3 times in 650 ml PBS pH 7.2, 0.1 % w/v bovine serum albumine (Sigma), 0.001 % bovine gamma globulin (Sigma). The particles are heat treated for 7 days at 37°C. The particles are washed in 0.01 M phosphate buffer twice. The particles are diluted to 0.5 g per 1 in PBS pH 7.2, 0.5 % human serum albumine (Sigma).

Example 1

Detection and quantification of antigen (total IgE)

Determination of total IgE antibodies, according to the invention, was conducted on Ciba Corning ACS:180 Benchtop Immunoassay analyzer described in Clinical Chemistry, 36/9, 1598-1602 (1990), using the following protocol:

50 µl of sample and 50 µl biotinylated anti-IgE are dispensed by the sample probe into the cuvette. The cuvette reaches the first reagent probe R1, where 100 µl paramagnetic particles with immobilised monoclonal anti-IgE antibody (ALK Laboratories A/S, Hørsholm, Denmark) specific against the IgE Fc domain are dispensed together

with 200 µl of streptavidin-acridinium ester label (ALK Laboratories A/S, Hørsholm, Denmark). The cuvette moves down the track to the magnets and wash station. Wash with 750 µl deionized water is performed twice. After completion of the wash cycle the particles are resuspended in 300 µl 0.5 g/l H_2O_2 in 0.1 M HNO_3. The cuvette enters the luminometer chamber and in front of the photomultiplier 300 µl 25 mM NaOH solution is added and the photons of light emitted are measured and quantitated and expressed as relative light units (RLU). The amount of RLU is directly proportional to the amount of the IgE in the sample. The time from sample dispension to first result is 15 min and a new result follows every 20 second. Results were expressed as RLU experiment/RLU background, where RLU background was the chemiluminescent reaction observed in the absence of total IgE.

Nine Total IgE standards, calibrated in Magic Lite Total IgE Kit (ALK Laboratories A/S, Hørsholm, Denmark) against WHO 2nd IRP no 75/502 for human serum IgE, were assayed using the protocol described above and it is shown that 0.1 IU/ml of total serum IgE could be detected (as determined by background x 10 standard deviations), see Figure 2.

Example 2

Detection and quantification of specific antibody (Specific IgE)

Determination of Phleum pratense specific IgE antibodies (timothy grass specific IgE, according to the invention, was conducted on Ciba Corning ACS:180 Benchtop Immunoassay analyzer described in Clinical Chemistry, 36/9, 1598-1602 (1990), using the following protocol:

50 µl of sample and 50 µl biotinylated Phleum pratense are dispensed by the sample probe into the cuvette. The cuvette reaches the first reagent probe R1, where 100 µl paramagnetic particles with immobilised monoclonal anti-IgE antibody (ALK Laboratories A/S, Hørsholm, Denmark) specific against the IgE Fc domain are dispensed together with 200 µl of streptavidin-acridinium ester label (ALK Laboratories A/S, Hørsholm, Denmark). The cuvette moves down the track to the

magnets and wash station. Wash with 750 µl deionized water is performed twice. After completion of the wash cycle the particles are resuspended in 300 µl 0.5 g/l H_2O_2 in 0.1 M HNO_3. The cuvette enters the luminometer chamber and in front of the photomultiplier 300 µl 25 mM NaOH solution is added and the photons of light emitted are measured and quantitated and expressed as relative light units (RLU). The amount of RLU is directly proportional to the amount of the IgE in the sample. The time from sample dispension to first result is 15 min and a new result follows every 20 second. Results were expressed as RLU experiment/RLU background, where RLU background was the chemiluminescent reaction observed in the absence of total IgE.

Ten Phleum pratense specific IgE standards, calibrated in Magic Lite SQ Specific IgE Kit (ALK Laboratories A/S, Hørsholm, Denmark) against clinically characterised Phleum pratense allergic patients samples and expressed as SU/ml (Standardised Units), were assayed using the protocol described above and it is shown that between 1.43 and 800 SU/ml of Phleum pratense specific IgE can be measured as in Magic Lite SQ Specific IgE assay, see Figure 3.

Example 3

Quantification of specific IgE against WHO Total IgE reference

Quantification of specific IgE antibodies in serum samples were performed with reference to total IgE antibody or specific IgE antibody using the identical assay protocols as described in example one and two, respectively.

Thirtyfive patient samples were assayed for Phleum pratense specific IgE along with 10 Phleum pratense specific IgE standards, calibrated in Magic Lite SQ Specific IgE Kit (ALK Laboratories A/S, Hørsholm, Denmark) against clinically characterised Phleum pratense allergic patient samples and expressed as SU/ml (protocol described in example 2).

Nine standards of IgE WHO 2nd IRP No. 75/502 (National biological standard board) were assayed in the same run against total IgE

(protocol described in example 1).

Figure 4 provides a comparison of the two dose response curves of total IgE assay and Phleum pratense specific IgE assay respectively. Parallel line test showed no significant difference in slopes between the two dose response curves, indicating that specific IgE in patient samples could be calibrated against the WHO Total IgE standard and expressed in IU/ml.

Figure 5 provides a comparison of results from the 35 patient samples calibrated against WHO Total IgE standards (expressed in IU/ml) or Phleum pratense specific IgE standards (expressed in SU/ml) and it shows very good correlation between the two units.

The concentration (dose) of the unknown sample was calculated using a cubic-free spline interpolation after a log vs. log transformation of signal/background and concentration (dose), respectively.

It was calculated from the linear regression line that one SU (Standardised Unit) corresponds to 0.14 International Unit (IU).

In conclusion allergen specific IgE can be measured using the embodiment of the present invention and calibrated directly from the total IgE assay of a WHO IgE calibrated standard curve.

Example 4

Total IgE method comparison

Thirtythree patients samples were measured for total serum IgE in the Magic Lite Total IgE kit (ALK Laboratories A/S, Hørsholm, Denmark). The assay was performed according to the manufacturer's instruction. The same samples were measured for total serum IgE on the ACS:180 according to the protocol described in example 1.

Figure 6 provides a scatterplot for the method comparison for measuring total serum IgE. A correlation with r = 0.90 was found.

Example 5

Specific IgE method comparison

Thirtyfive patients samples were measured for Phleum pratense specific IgE in the Magic Lite SQ Specific IgE kit (ALK Laboratories A/S, Hørsholm, Denmark). The assay was performed according to the manufacturer's instruction. The same samples were measured for Phleum pratense specific IgE on the ACS:180 according to the protocol described in example 2.

Figure 7 provides a scatterplot for the method comparison for measuring Phleum pratense specific IgE. A correlation with r = 0.80 was found.

Example 6

Comparison of assay precision

The within-run imprecision for ACS total IgE using the protocol described in example 1, was compared to that of Magic Lite Total IgE. Patient samples were run in replicates of three in the assays described in example 4. The pooled within-run coefficient of variation (CVpwr) was calculated according to Krouwer and Rabinowitz, Clinical Chemistry, 30, 290 (1984). The following results were obtained:

	Magic Lite	ACS
%CVpwr	4.69	2.75
Min %CV	0.80	0.70
Max %CV	11.70	8.40

As seen from the results, automation and minimation of operating steps significantly improves the precision of the analysis (by F-test: F=1.71, p=0.049).

Example 7

Comparison of total and specific IgE in patient samples:

The samples measured for Phleum pratense specific IgE on the ACS:180 according to the protocol described in example 2 and calibrated against total IgE reference (WHO 75/502) as described in example 3, were also analysed for total IgE as described in example 1. The ratio between measured specific IgE and total IgE was calculated on each sample and expressed as % ratio (spec. IU/total IU*100).

The following results were obtained:

No	Phleum pratense Specific IgE IU/ml	Total IgE IU/ml	% Ratio
1	0.973	208.300	0.467
2	0.000	153.330	0.000
3	0.000	25.119	0.000
4	0.000	43.980	0.000
5	0.082	51.997	0.158
6	0.586	30.807	1.902
7	9.130	20.796	43.903
8	2.125	168.771	1.259
9	1.545	321.553	0.480
10	0.000	299.105	0.000
11	1.664	248.660	0.669
12	8.553	46.635	18.340
13	19.427	234.858	8.272
14	0.000	178.304	0.000
15	2.380	428.987	0.555
16	4.342	112.457	3.861
17	3.975	173.793	2.287
18	0.220	206.580	0.106
19	0.158	298.630	0.053
20	4.936	17.116	28.839
21	0.376	146.939	0.256
22	4.865	23.521	20.684
23	3.115	281.804	1.105
24	0.017	17.937	0.095

25	3.446	160.651	2.145
26	1.485	167.427	0.887
27	2.763	124.309	2.223
28	3.705	247.679	1.496
29	1.289	196.314	0.657
30	8.967	416.528	2.153
31	0.355	29.784	1.192
32	15.173	290.670	5.220
33	2.141	63.847	3.353
34	3.929	96.951	4.053
35	0.154	> 620	ND

Example 7 (continued)

As seen from the results the Phleum pratense specific IgE assay was able to measure as low as 0.05 % of Phleum pratense specific IgE out of total IgE. Low relative amounts of Phleum pratense specific IgE indicates that other allergen specificities are present in the samples. Up to 44 % of total IgE was found in one patient sample to be specific against Phleum pratense. No correlation was found between total IgE concentration and Phleum pratense specific IgE concentration as seen in figure 8.

Example 8

Determination of serum total IgA antibodies using paramagnetic particles and avidin-acridinium ester label.

The determination of total IgA antibodies was assayed using the following protocol:

25 µl of patient sample or calibrator was pipetted into a 12x75 mm test tube. To each tube 50 µl of biotinylated polyclonal anti-IgA antibody DAKO E484 (supplied by DAKO, Glostrup, Denmark) in 0.05 M phosphate buffer, pH 7.4, containing 0.1 % sodium azide, 0.01 % Tween® 20 and 0.1 % human serum albumin was added and reacted for 15 minutes at ambient temperature. 400 µl slurry of paramagnetic particles with immobilised polyclonal anti-IgA antibody DAKO A 262, (supplied by DAKO, Glostrup, Denmark) was added to each tube and incubated for 5 minutes. After this second incubation 50 µl of streptavidin-acridinium ester label diluted in the same buffer as described above was added to each tube and incubated for a further 5 minutes at ambient temperature. The paramagnetic particles were washed twice with a 0.2 M phosphate buffer, pH 7.4, containing 0.1 % Tween® 20, after separating the magnetic particles from the liquid by a magnetic base separator and vortexing the separated particles with the washing buffer as described above. The contents of the tubes were finally measured in the luminometer, where light emitted at 426 nm, was quantitated and expressed as relative light units (RLUs).

Total IgA standards calibrated against WHO No. 67/86 for human serum IgA DAKO X908 (supplied by DAKO, Glostrup, Denmark) were assayed using the above described protocol.

Standards:

Concentration (µg/ml)	RLUs
0	370937
0.02	417000
0.23	557563
2.32	1252260
23.2	1872357

It should be apparent to one having ordinary skill in the art that many variations are possible without departing from the spirit and scope of the invention.

Claims

1. A method of detecting an antibody in a sample using a chemiluminescent labelling compound, comprising

a) mixing a ligand antigen, antibody, or hapten bound to biotin or a functional derivative thereof; an antibody directed against the antibody to be detected bound to paramagnetic particles; and a chemiluminescent acridinium compound bound to avidin, streptavidin or a functional derivative thereof with the sample to form a solid phase bound complex,

b) magnetically separating the solid phase from the liquid phase,

c) initiating the chemiluminescent reaction, and analysing the separated solid phase for the presence of chemiluminescent complex, wherein the presence of chemiluminescence is an indication of the presence of said antibody in said sample.

2. A method according to claim 1, comprising

i) mixing the ligand antigen, antibody, or hapten bound to biotin or a functional derivative thereof with the sample and the antibody directed against the antibody to be detected bound to paramagnetic particles to form a first solid phase complex,

ii) adding a chemiluminescent acridinium compound covalently bound to avidin, streptavidin or a functional derivative thereof to form a second solid phase complex,

iii) magnetically separating the solid phase from the liquid phase;

iv) initiating the chemiluminescent reaction, and analysing the separated solid phase for the presence of chemiluminescent complex.

3. A method according to claim 1 or 2, wherein the antibody in the sample is a specific immunoglobulin selected from the group consisting of specific IgA, IgD, IgE, IgG, IgM, and isotypes thereof, and the ligand antigen, antibody, or hapten is a specific allergen.

4. A method according to claim 3, wherein the specific immunoglobulin is a specific IgE.

5. A method according to claim 1 or 2, wherein the antibody in the sample is a class of immunoglobulins selected from the group consisting of total IgA, total IgD, total IgE, total IgG, total IgM, and isotypes thereof, and the ligand antigen, antibody or hapten is an antibody directed against said class of immunoglobulins.

6. A method according to claim 5, wherein the class of immunoglobulins is total IgE.

7. A method according to any one of the preceding claims, wherein the antibody directed against the antibody to be detected bound to paramagnetic particles is selected from the group consisting of polyclonal antibodies, monoclonal antibodies including recombinant antibodies, fragmented antibodies; preferably a monoclonal mouse anti-immunoglobulin.

8. A method according to any one of the preceeding claims, wherein the chemiluminescent acridinium compound is N-hydroxysuccinimide dimethylacridinium ester covalently bound to avidin, streptavidin or a functional derivative thereof.

9. A method of measuring the concentration and/or the relative contents of a specific antibody in a sample, wherein the measured light emission of a separated solid phase comprising a captured specific antibody coupled to a chemiluminescent label is compared with the measured light emission obtained in a truly parallel reference immuno-assay wherein the total contents of the class of antibodies in the sample to which said specific antibody belongs is measured.

10. A method according to claim 9, wherein the light emission of the separated solid phase comprising the captured specific antibody coupled to a chemiluminescent label is obtained by

a) mixing a ligand antigen or hapten towards which the specific antibody to be measured is directed bound to biotin or a functional

derivative thereof; an antibody directed against the constant portion of the antibody to be measured bound to paramagnetic particles; and a chemiluminescent acridinium compound bound to avidin, streptavidin or a functional derivative thereof with the sample to form a solid phase complex,

b) magnetically separating the solid phase from the liquid phase,

c) initiating the chemiluminescent reaction and measuring the light emission,

and the reference immuno-assay for the class of antibodies to which said specific antibody belongs comprises

a) mixing a ligand antibody directed against said class of antibodies to be measured bound to biotin or a functional derivative thereof; an antibody directed against the constant portion of the class of antibodies to be measured bound to paramagnetic particles; and a chemiluminescent acridinium compound bound to avidin, streptavidin or a functional derivative thereof with the sample to form a solid phase complex,

b) magnetically separating the solid phase from the liquid phase,

c) initiating the chemiluminescence reaction and measuring the light emission.

11. A method according to claim 9, wherein the light emission of the separated solid phase comprising the captured specific antibody coupled to a chemiluminescent label is obtained by

i) mixing the ligand antigen or hapten towards which the specific antibody to be measured is directed bound to biotin or a functional derivative thereof with the sample and the antibody directed against the constant portion of the antibody to be measured bound to paramagnetic particles to form a first solid phase complex,

ii) adding a chemiluminescent acridinium compound covalently bound to avidin, streptavidin or a functional derivative thereof to form a

second solid phase complex,

iii) magnetically separating the solid phase from the liquid phase,

iv) initiating the chemiluminescent reaction, and measuring the light emission,

and the reference immuno-assay for the class of antibodies to which said specific antibody belongs comprises

i) mixing a ligand antibody directed against said class of antibodies to be measured bound to biotin or a functional derivative thereof with the sample and the antibody directed against the constant portion of the class of antibodies to be measured bound to paramagnetic particles to form a first solid phase complex,

ii) adding a chemiluminescent acridinium compound covalently bound to avidin, streptavidin or a functional derivative thereof to form a second solid phase complex,

iii) magnetically separating the solid phase from the liquid phase,

iv) initiating the chemiluminescent reaction and measuring the light emission.

12. A method according to claim 9, 10 or 11, wherein the specific antibody to be measured in the sample is a specific immunoglobulin selected from the group consisting of IgA, IgD, IgE, IgG, IgM, and isotypes thereof, and the ligand antigen, antibody, or hapten directed against the variable portion of said antibody is an allergen, and the class of antibodies is a class of immunoglobulins selected from the group consisting of total IgA, total IgD, total IgE, total IgG, total IgM, and isotypes thereof, and the ligand antigen, antibody or hapten is an antibody directed against said class of immunoglobulins.

13. A method according to claim 12, wherein the specific immunoglobulin is a specific IgE, and the class of antibodies is total IgE.

14. A method according to claim 10 or 11, wherein the antibody directed against the antibody to be measured bound to paramagnetic particles is selected from the group consisting of polyclonal antibodies, monoclonal antibodies including recombinant antibodies, fragmented antibodies, preferably a monoclonal mouse anti-immunoglobulin.

15. A method according to any one of the preceeding claims, wherein the chemiluminescent acridinium compound is N-hydroxysuccinimide dimethylacridinium ester covalently bound to avidin, streptavidin or a functional derivative thereof.

Figure 1

Specific IgE Detection Assay:
Solid phase (anti-IgE)
Sample (IgE)
Biotinylated-allergen
BIOTIN
AVIDIN-DMAE
BIOTIN-AVIDIN-DMAE
Total IgE Detection Assay:
Solid-phase (anti-IgE)
Sample (IgE)
Biotinylated-antibody (anti-IgE)
BIOTIN
AVIDIN-DMAE
BIOTIN-AVIDIN-DMAE

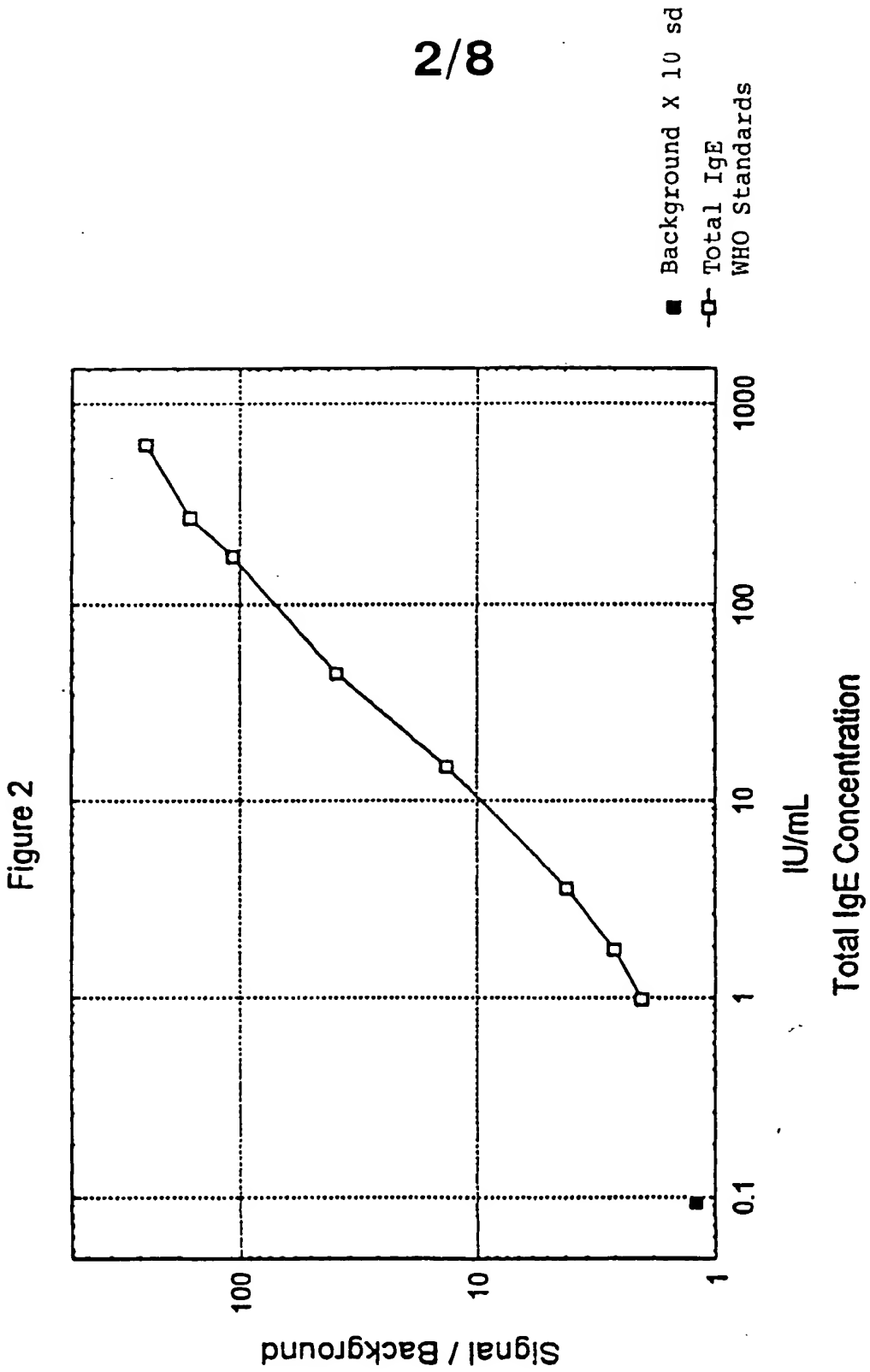
Background X 10 sd
Total IgE
WHO Standards
Signal / Background
100
10
1
0.1
1
10
100
1000
IU/mL
Total IgE Concentration

Figure 2

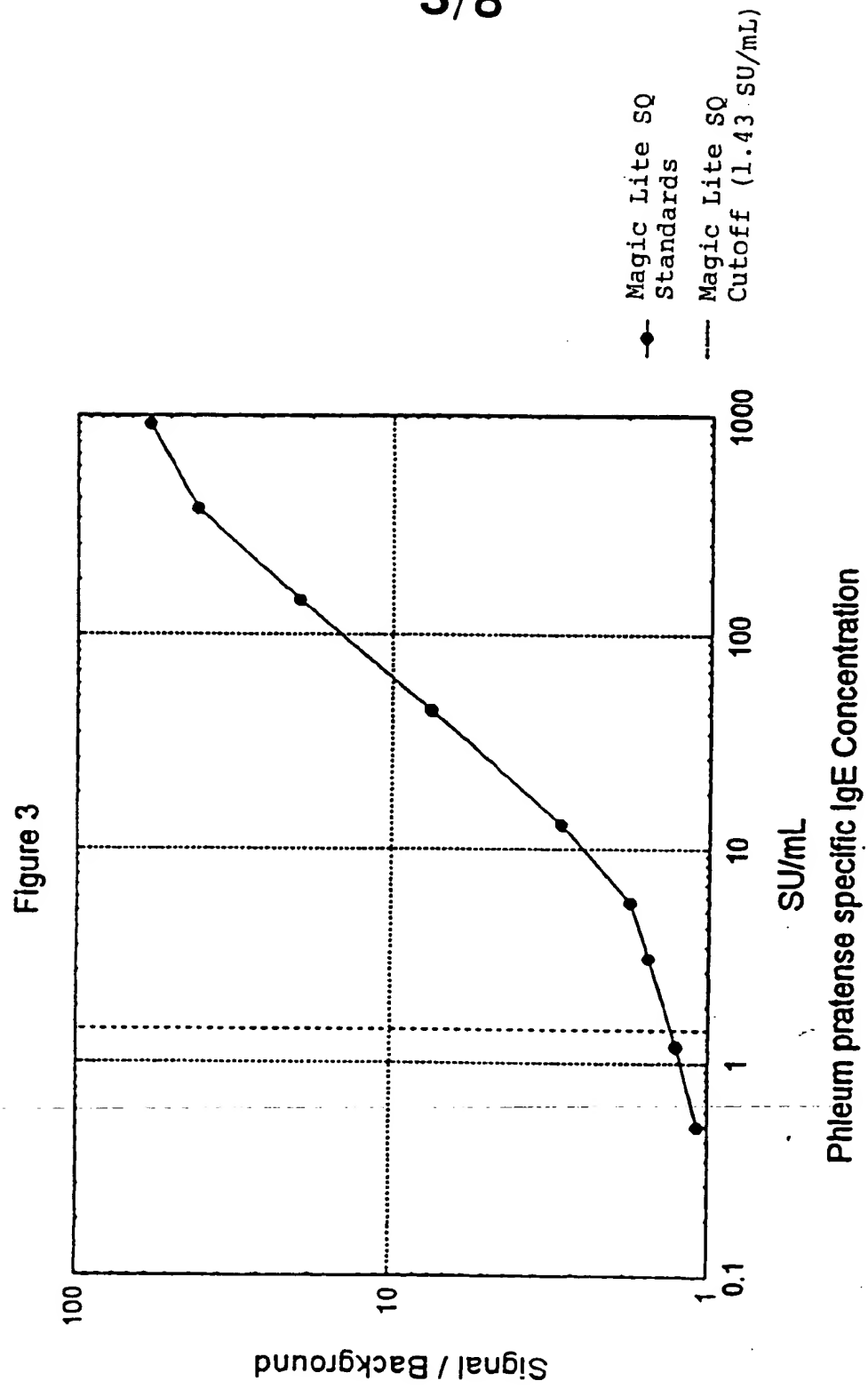
Magic Lite SQ Standards
Magic Lite SQ Cutoff (1.43 SU/mL)
Signal / Background
100
10
1
0.1
1
10
100
1000
SU/mL
Phleum pratense specific IgE Concentration

Figure 3

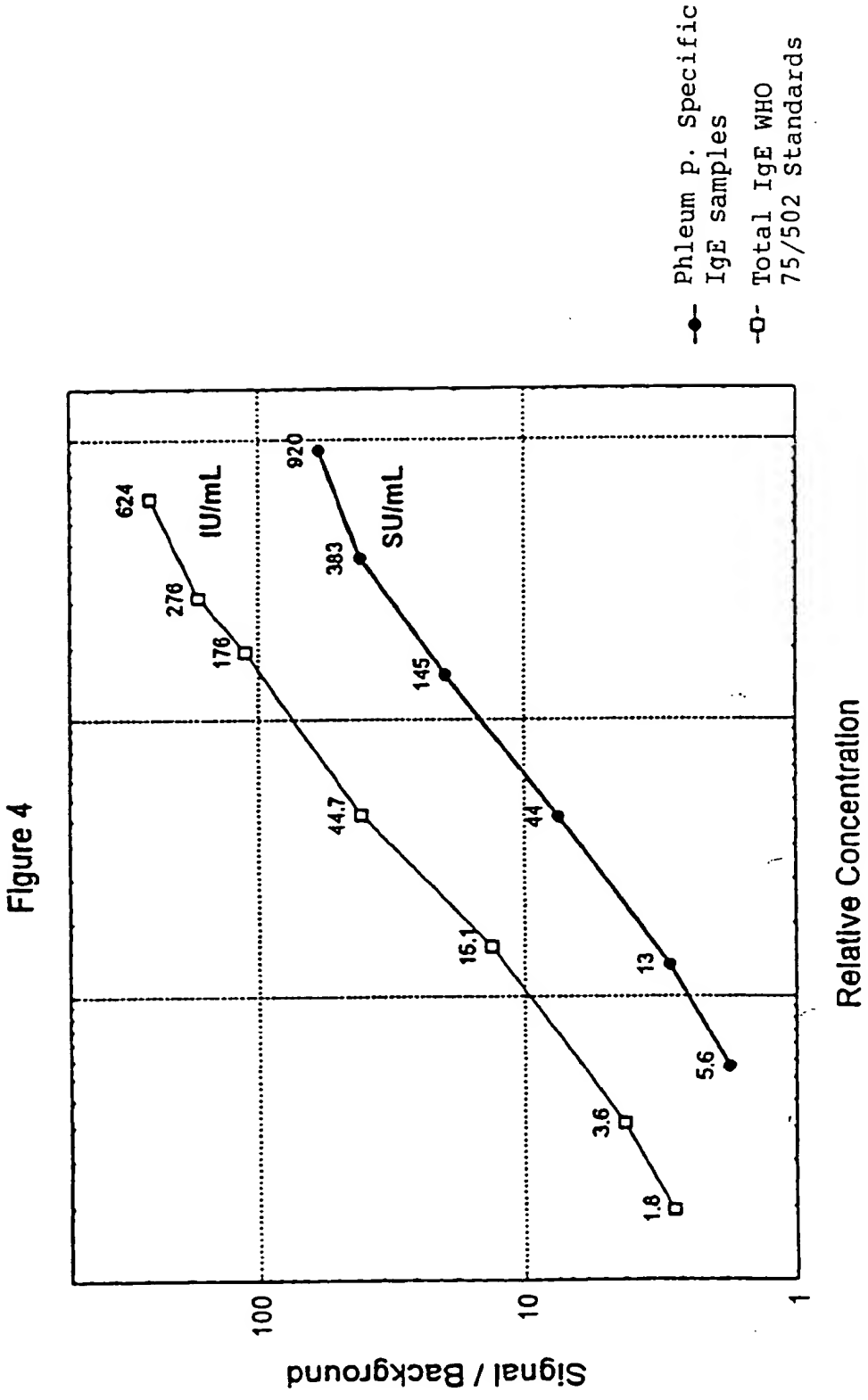
Phleum p. Specific IgE samples
Total IgE WHO 75/502 Standards
Signal / Background
100
10
1
Relative Concentration
IU/mL
624
276
176
44.7
16.1
3.6
1.8
SU/mL
920
383
145
44
13
5.6

Figure 4

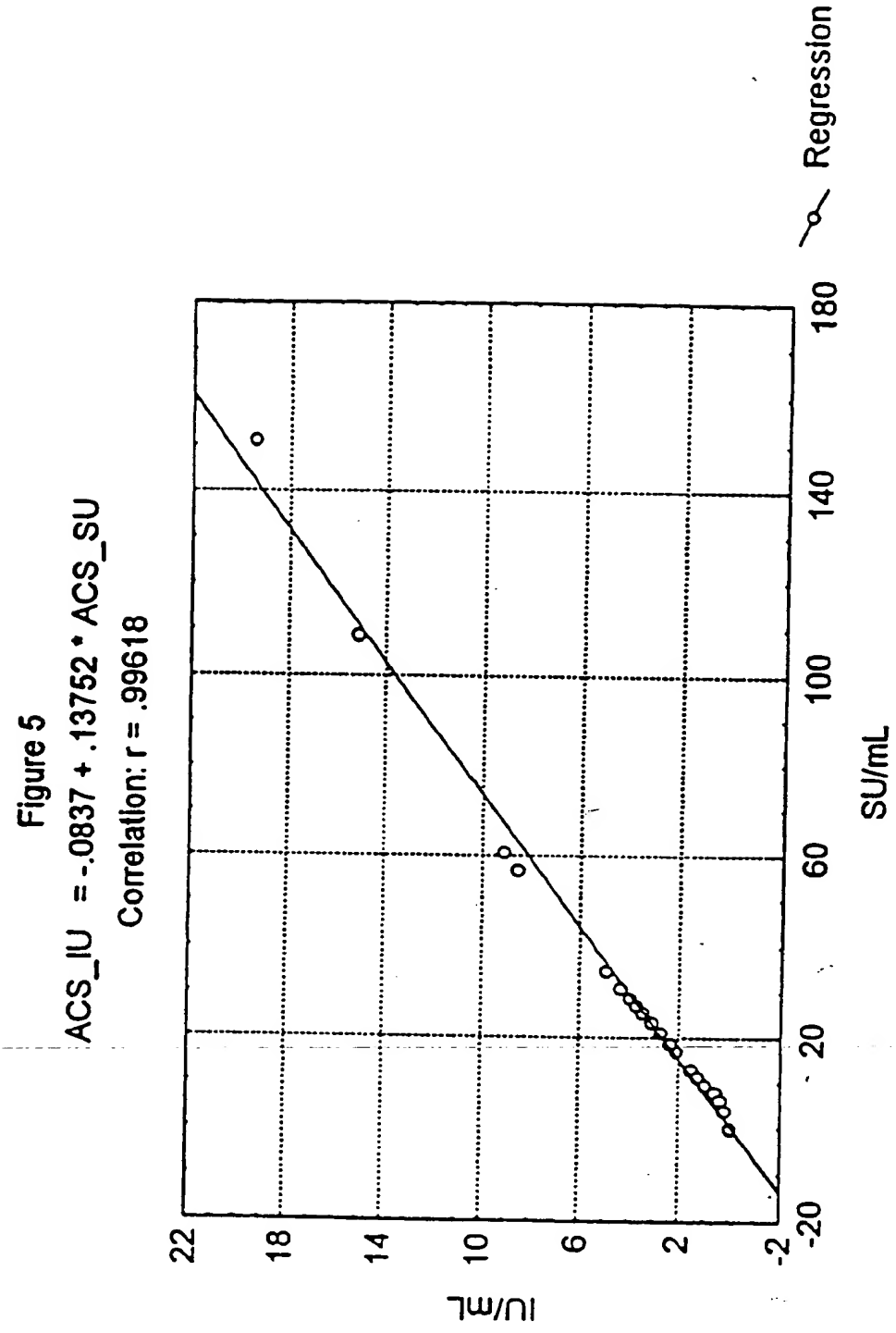

Figure 5
ACS_IU = -.0837 + .13752 * ACS_SU
Correlation: r = .99618

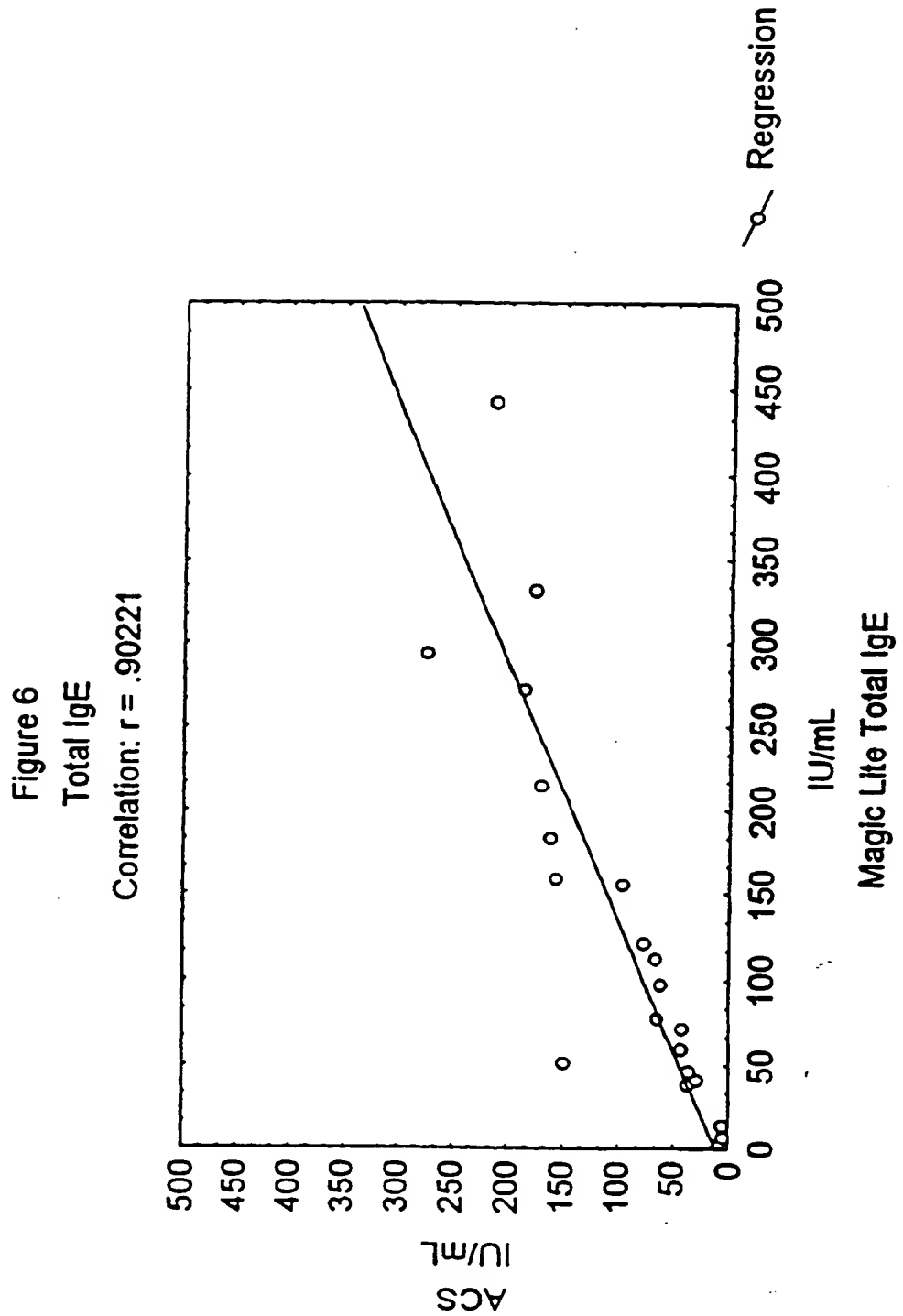

Figure 6
Total IgE
Correlation: r = .90221

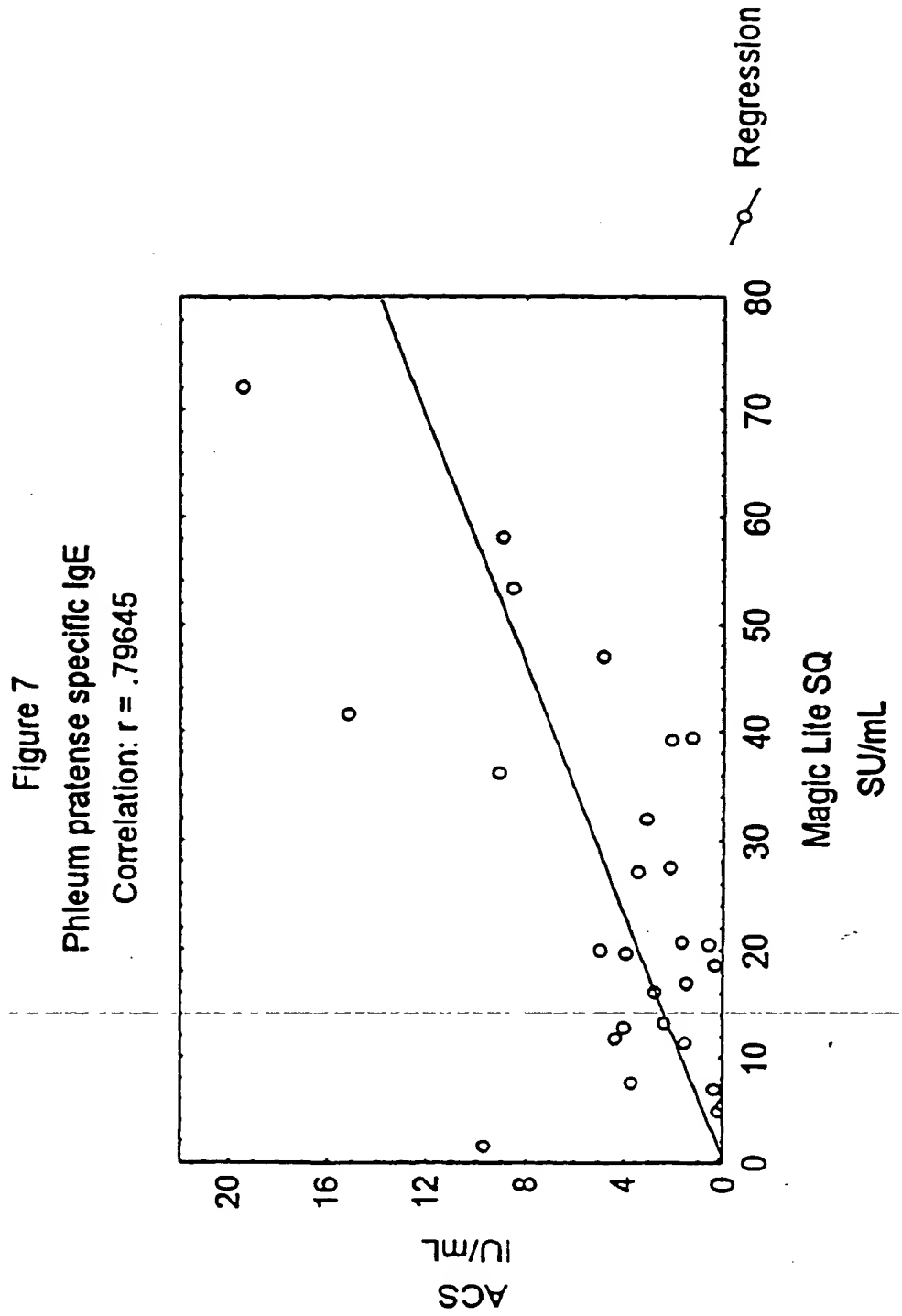

Figure 7
Phleum pratense specific IgE
Correlation: r = .79645

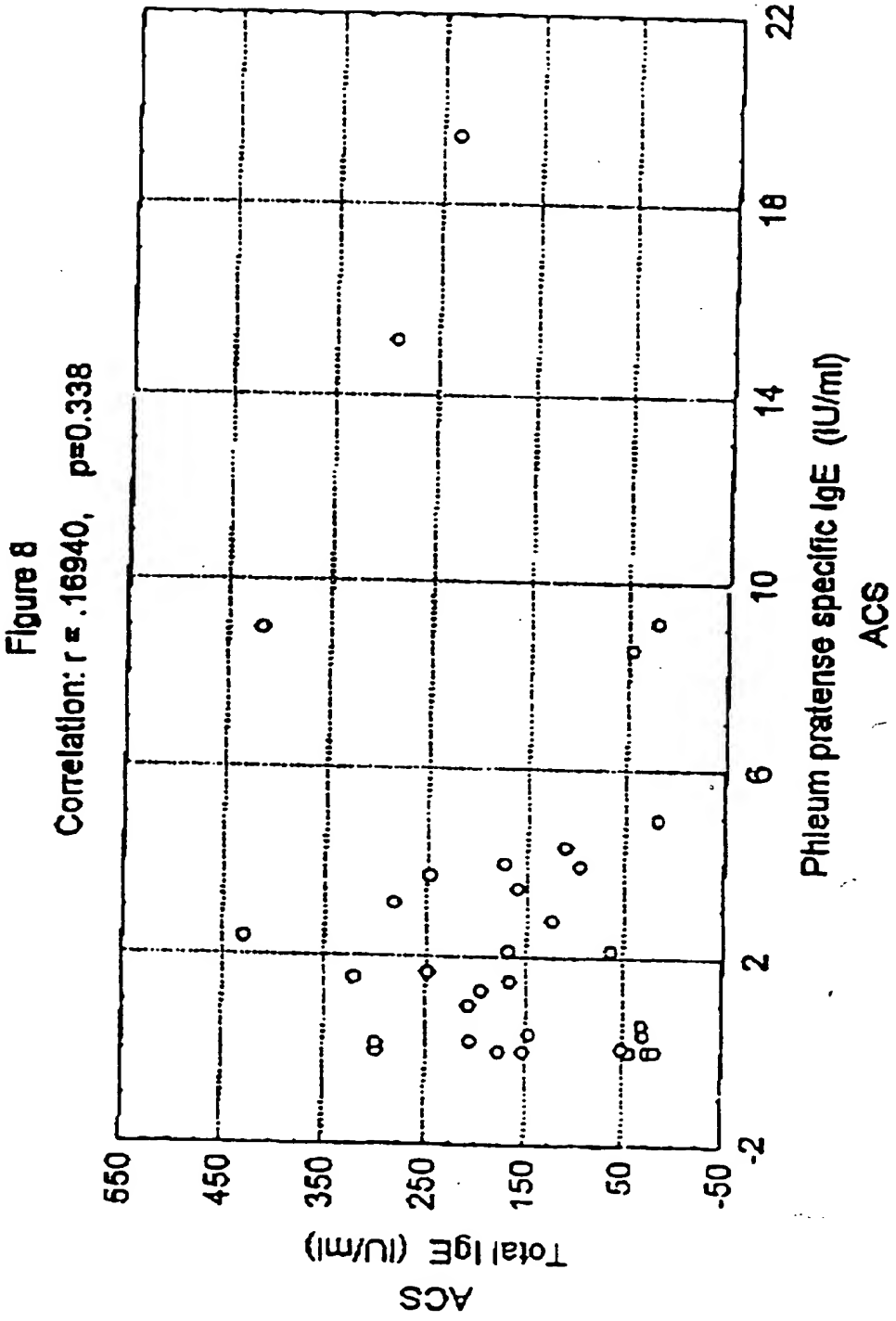
Figure 8
Correlation: r = .16940, p=0.338
ACS
Total IgE (IU/ml)
550
450
350
250
150
50
-50
-2
2
6
10
14
18
22
Phleum pratense specific IgE (IU/ml)
ACS

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 93/00373

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: G01N 33/543, G01N 33/68
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CLINICAL AND EXPERIMENTAL ALLERGY, Volume 22, 1992, J. Kleine-Tebbe et al, "Comparison between MAGIC LITE® - and CAP® system: two automated specific IgE antibody assays", page 475 - page 484, see p 476 and ref. 5, 6 and 15	1-15
Y	METHODS IN ENZYMOLOGY, Volume 73, 1981, J-L Guesdon et al, "Magnetic Solid-Phase Enzyme Immunoassay for the Quantitation of Antigens and Antibodies: Application to Human Immunoglobulin E" page 471	1-15

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 February 1994	21 -02- 1994
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carl Olof Gustafsson Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 93/00373

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF IMMUNOLOGICAL METHODS, Volume 116, 1989, M. Sakaguchi et al, "Measurement of antigen-specific mouse IgE by a fluorometric reverse (IgE-capture) ELISA", page 181 - page 187, fig 1, pages 182-183 --	1-15
Y	National Library of Medicine, File Medline, NLM accession no. 87281685, Hart, RC: "The use of acridinium ester-labelled streptavidin in immuno-assays", J Immunol Methods 1987 Jul 16;101(1):91-6 --	1-15
A	National Library of Medicine, File Medline, NLM accession no. 89102665, Bagazgoitia, FJ: "Effect of surfactants on the intensity of chemi-luminescence emission from acridinium ester labelled proteins", J Biolumin Chemilumin 1988 Jul-Sep;2(3): 121-8 --	1-15
A	METHODS IN ENZYMOLOGY, Volume 184, 1990, C. J. Strasburger et al, "Two-Site and Competitive Chemiluminescent Immunoassays" page 481 - page 496 -- --------	1-15

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INTERNATIONAL SEARCH REPORT

Inter .onal Application No
PCT/US 98/06774

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/566 G01N33/68

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 97 20859 A (IDEXX LAB INC) 12 June 1997 see claims 6,7,10-14,16-27,29,30 see page 1, line 13 - line 21 see page 6, line 15 - line 16 see page 15, line 3 - line 16 see page 29, line 12 - line 25 ---	1-39
X	WO 95 16203 A (GENENTECH INC ;TAI WAI FEI DAVID (US); LOWE JOHN (US); JARDIEU PAU) 15 June 1995 see claims 1,8,14-16 see page 4, line 18 - page 6, line 9 see page 12, line 25 - page 13, line 5 see page 13, line 14 - line 19 see page 23, line 9 - page 24, line 38 --- -/--	1-39

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"E" earlier document but published on or after the international filing date

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 July 1998	28/07/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Routledge, B

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INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 98/06774

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication.where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 29696 A (QUIDEL CORP) 22 December 1994 cited in the application see claims see the whole document	20-39